

Klotho Reduction in Alveolar Macrophages Contributes to Cigarette Smoke Extract-induced Inflammation in Chronic Obstructive Pulmonary Disease^{*S}

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Background: The role of Klotho in airway inflammation in COPD remains unclear.

Results: Klotho expression was reduced in alveolar macrophages in peripheral lungs and PBMCs of smokers with and without COPD. Klotho regulates macrophage inflammation via NF- κ B pathway.

Conclusion: Klotho plays a role in sustained inflammation of the lungs.

Significance: These findings suggest Klotho may have therapeutic implications in COPD.

Abnormal inflammation and accelerated decline in lung function occur in patients with chronic obstructive pulmonary disease (COPD). Klotho, an anti-aging protein, has an anti-inflammatory function. However, the role of Klotho has never been investigated in COPD. The aim of this study is to investigate the possible role of Klotho by alveolar macrophages in airway inflammation in COPD. Klotho levels were assessed in the lung samples and peripheral blood mononuclear cells of non-smokers, smokers, and patients with COPD. The regulation of Klotho expression by cigarette smoke extract (CSE) was studied *in vitro*, and small interfering RNA (siRNA) and recombinant Klotho were employed to investigate the role of Klotho on CSE-induced inflammation. Klotho expression was reduced in alveolar macrophages in the lungs and peripheral blood mononuclear cells of COPD patients. CSE decreased Klotho expression and release from MH-S cells. Knockdown of endogenous Klotho augmented the expression of the inflammatory mediators, such as MMP-9, IL-6, and TNF- α , by MH-S cells. Exogenous Klotho inhibited the expression of CSE-induced inflammatory mediators. Furthermore, we showed that Klotho interacts with I κ B α of the NF- κ B pathway. Dexamethasone treatment increased the expression and release level of Klotho in MH-S cells. Our findings suggest that Klotho plays a role in sustained inflammation of the lungs, which in turn may have therapeutic implications in COPD.

Chronic obstructive pulmonary disease (COPD)³ is a major cause of disability, morbidity, and mortality in smokers. It is

characterized by destruction of the alveolar wall, decline in lung function, and chronic inflammatory response (Global Initiative for COPD (GOLD) guidelines). Cigarette smoking is implicated as a major risk factor for the development of COPD because ~90% of patients with COPD are smokers (1). Furthermore, COPD is linked with the aging process of the lungs due to its direct encounter with inhaled cigarette smoke-derived oxidants and free radicals as well as other organic constituents (2–4). It is well known that abnormal inflammatory responses to smoking in patients with COPD are due to chronic inflammatory effects incited by cigarette smoking (5, 6). However, due to the complex nature of the mechanisms of the inflammatory processes in COPD (5, 6), the precise molecular mechanisms whereby CSE triggers abnormal and sustained lung inflammation and the aging process still remain unclear. Although cigarette smoking has been proved to be the most significant risk factor for COPD, only 10–20% of chronic smokers develop this disease (7). This fact indicates that development of COPD is affected by factors other than smoking, such as genetic factors.

The *klotho* gene was discovered by Kuro-o *et al.* (8) during the development of hypertensive transgenic mouse models. Mice homozygous for the transgene demonstrate various phenotypes resembling premature aging syndrome, *i.e.* a short life span, arteriosclerosis, osteoporosis, skin atrophy, and pulmonary emphysema. Previous studies have shown that *klotho* mRNA is expressed mainly in the brain, kidney, reproductive organs, pituitary gland, and parathyroid glands (8, 9). We recently demonstrated that Klotho is also expressed in the lung and is down-regulated in the tissue in COPD lung (10).

The pathophysiology of COPD is multifactorial, with an inflammatory cell profile that includes macrophages, neutrophils, and T lymphocytes (5, 6, 11, 12). Macrophages are derived from monocytes and are suggested to be the main orchestrators of the chronic inflammatory responses seen in patients with COPD (13). Macrophages from patients with COPD release higher levels of pro-inflammatory cytokines (TNF- α and IL-6) and matrix metalloproteases (MMP-9) compared with non-smoking control subjects (13, 14). The expression of matrix

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^S This article contains supplemental Table S1.

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³ The abbreviations used are: COPD, chronic obstructive pulmonary disease; CSE, cigarette smoke extract; PBMC, peripheral blood mononuclear cell; NAC, N-acetylcysteine.

TABLE 1**The baseline characteristic of the patients**

The abbreviations used are as follows: FEV₁/FVC, forced expiratory volume 1/forced vital capacity; DLCO (SB), diffusing capacity for carbon monoxide (single breath); DLCO/AV, diffusing capacity for carbon monoxide/alveolar volume; BMI, body mass index.

	Healthy controls	Healthy smokers	COPD
No. of males/females	22 (8/14)	22 (20/2)	15 (13/2)
Age	61.6 ± 11.1	66.1 ± 11.5	64.8 ± 9.3
FEV ₁ /FVC (%)	77.5 ± 4.5	76.7 ± 4.7	57.6 ± 6.1 ^a
FEV ₁ (%)	92.9 ± 16.8	92.8 ± 16.5	64.2 ± 13.1 ^a
DLCO (SB)	92.9 ± 16.8	87.9 ± 16.0	64.6 ± 26.0 ^a
DLCO/AV	98.0 ± 20.1	94.6 ± 15.3	73.7 ± 29.1 ^a
BMI	24.2 ± 3.1	24.3 ± 2.9	22.7 ± 3.1
Smoking (pack years)	0	828.2 ± 767.0	820.0 ± 379.8

^a Data are significantly lower in the COPD group than in healthy smoking and control groups.

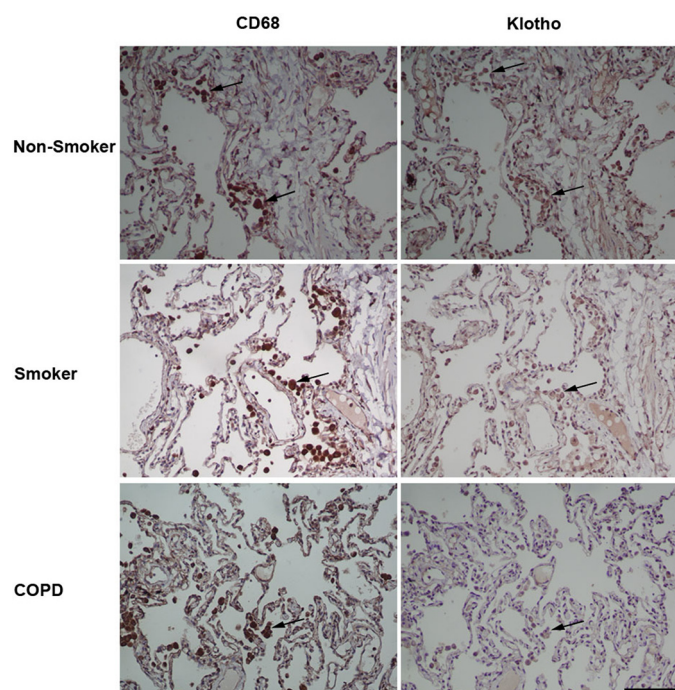


FIGURE 1. Decreased staining of Klotho in lung alveolar macrophages of smokers with and without COPD. Serial lung sections from nonsmokers ($n = 22$) and smokers with ($n = 15$) and without COPD ($n = 22$) were stained with anti-Klotho antibody and anti-CD68 antibody. Dark brown color represents the presence of CD68 and Klotho. Bar = 100 μ m.

metalloproteases increases with the development of emphysema in the lungs of Klotho ($KL^{-/-}$) mice (15). In light of this, we hypothesized that Klotho may prevent airway inflammation by alveolar macrophages in COPD. In this study, we therefore studied the expression of Klotho in alveolar macrophages in the peripheral lungs of non-smokers, smokers, and patients with COPD. In addition, the role of Klotho in the regulation of pro-inflammatory cytokine release was also investigated.

Materials and Methods

Ethics Statement—Written informed consents were obtained from all patients. The Medical Ethical Committee of the First Affiliated Hospital of Nanjing Medical University approved the study protocol (2013-SRFA-037). All experiments were performed in accordance with relevant guidelines and regulations.

Lung Tissue and Peripheral Blood Mononuclear Cells (PBMCs)—Lung tissues were obtained from lung resection tissue taken from 22 non-smokers, 22 normal smokers, and 15 smokers with COPD. The baseline characteristics of the patients are summarized in Table 1 (FEV₁/FVC, DLCO (SB), DLCO (AV), and BMI). A 10-ml blood sample was taken from each patient or volunteer with anticoagulant-containing syringes. PBMCs were separated by AccuSPIN (Sigma, Poole, UK) and suspended in RPMI 1640 medium containing heat-inactivated 10% FBS and L-glutamine. Experiments were performed after overnight incubation.

Alveolar Macrophages—Murine alveolar macrophage cell line MH-S (CRL-2019), purchased from the American Type Culture Collection (ATCC), Manassas, VA, was used. The MH-S cells were maintained in RPMI 1640 medium (ATCC) supplemented with 10% fetal bovine serum and 1% streptomycin/penicillin/glutamate solution (Gibco). The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO₂. Fresh media were added every 2–3 days. The cells were starved with the medium with 1% FBS 24 h before use.

Preparation of Aqueous Cigarette Smoke Extract—CSE was prepared using a modification to a previously modified method (16–18). One commercial Marlboro Red cigarette (0.8 mg of nicotine; 10 mg of tar; 10 mg of carbon monoxide) was combusted with a modified syringe-driven apparatus. A 10% CSE was prepared by bubbling smoke from one cigarette into 10 ml of culture medium supplemented with 1% FBS at a rate of one cigarette/min. The pH of the CSE was adjusted to 7.4, and the medium was sterile-filtered through a 0.22- μ m Millex-GS filter (Millipore, Watford, UK). CSE was freshly prepared for each experiment and diluted with culture medium containing 1% FBS immediately before use.

Cell Proliferation Analysis—Cell proliferation was determined using a CCK-8 kit (Cell Counting kit-8; Dojindo Laboratories, Kumamoto, Japan), according to the manufacturer's instructions. MH-S cells (5×10^3 cells/well in a 96-well plate) were incubated for 24 h in the presence or absence of Klotho. CCK-8 solution was added to each well and incubated at 37 °C for 2 h. Absorbance was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, Pittsburgh, PA).

Assessment of Senescence-associated β -Galactosidase Staining—MH-S cells were seeded in a 6-well plate with 2×10^3 cell/cm². After 2 days of culture, the medium was discarded; cells were rinsed one time with PBS; and 1 ml of fixative per well was added for 15 min and subsequently rinsed three times with PBS. Then 1 ml per well of working solution of β -galactosidase with X-Gal was placed, and the plate was maintained at 37 °C overnight (senescence-associated β -galactosidase staining kit from Beyotime, China). The cells were observed under an inverted microscope.

RNA Interference—MH-S cells were seeded on 6-well plates. For the *klotho* knockdown experiments, the cells were transiently transfected with 20 μ M *klotho* small-interfering RNA (siRNA1, siRNA2, and siRNA3) or negative control siRNA (NC siRNA) using LipofectamineTM 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. After 72 h, the protein expression of *klotho* was detected by Western blotting.

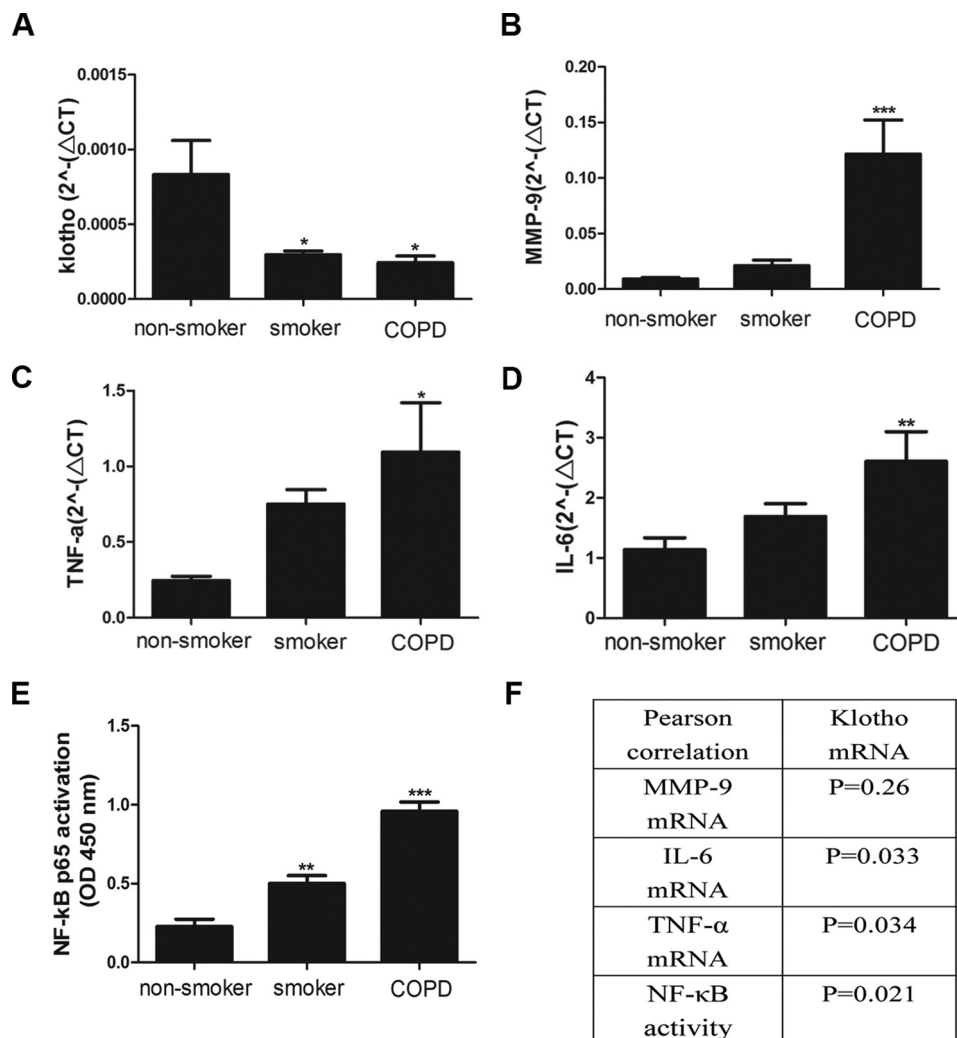


FIGURE 2. Decreased expression of Klotho and increased level of inflammation in PBMCs of smokers with and without COPD. PBMCs were collected from non-smokers ($n = 8$), smokers ($n = 8$), and COPD ($n = 8$). Total RNA of the cell was isolated after overnight incubation. *A*, *klotho* mRNA levels decreased in PBMCs from smokers and patients with COPD compared with nonsmokers. *B–D*, mRNA levels of MMP-9, IL-6, and TNF- α significantly increased in PBMCs of COPD patients as compared with those in healthy non-smokers. *E*, ELISA was used to detect nuclear NF- κ B DNA binding activity. NF- κ B activity significantly increased in PBMCs from smokers and patients with COPD compared with nonsmokers. *F*, summary of p values of Pearson correlation between *klotho* mRNA and MMP-9 mRNA or IL-6 mRNA or TNF- α mRNA or NF- κ B activity. *, $p < 0.05$; **, $p < 0.01$, and ***, $p < 0.001$ compared with non-smokers.

Immunohistochemistry—Two authors (Y. W. and W. G.) were blinded to the tissue of origin for the samples. They only knew the number of samples and used immunohistochemistry to determine the expression of Klotho and CD68 of samples. Buffered formalin (10%)-fixed paraffin-embedded lung sections (3 μ m thick) of nonsmokers, smokers, and COPD patients were deparaffinized using xylene and rehydrated in a graded ethanol series. Heat-induced antigen retrieval was performed in a microwave oven before immunohistochemical staining. After cooling and running under tap water, endogenous peroxidase activity was blocked by incubating in 3% hydrogen peroxide. To avoid the nonspecific background, blocking was done with 5% BSA/PBS solution for 1 h at room temperature. For the detection of the Klotho protein, the slides were incubated with polyclonal rabbit anti-Klotho (1:100 dilution) or anti-CD68 (1:100 dilution) at 4 °C overnight in a humidified chamber. After washing, sections were next incubated with peroxidase-conjugated goat anti-rabbit secondary antibody for 1 h at room temperature. The reactions were developed using a 3,3'-di-

aminobenzidine substrate kit, with hematoxylin as counter-stain. Each slide was evaluated under a microscope (Nikon, Tokyo, Japan) by one of the authors (L. L.). Tissue sections were scored for staining of the lining on a 0–5 scale (19) as follows: 0 = no staining; 1 = few of the cells positively stained; 2 = some (fewer than half) of the cells stained; 3 = approximately half of the cells stained; 4 = more than half of the cells stained; and 5 = all cells stained. For each section, the number of positively stained cells was counted in 20 fields.

Western Blot Analysis—Proteins were fractionated by SDS-PAGE and transferred to nitrocellulose or polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were blocked in 5% BSA for 1 h and then incubated with the indicated primary antibody at 4 °C overnight. Membranes were washed in Tris-buffered saline, 0.1% Tween 20 and incubated with secondary antibody at 20 °C for 1 h. Immunoblots were developed with an ECL Plus Western blotting detection system (Bio-Rad), as described previously (20, 21). The gray values of protein bands were

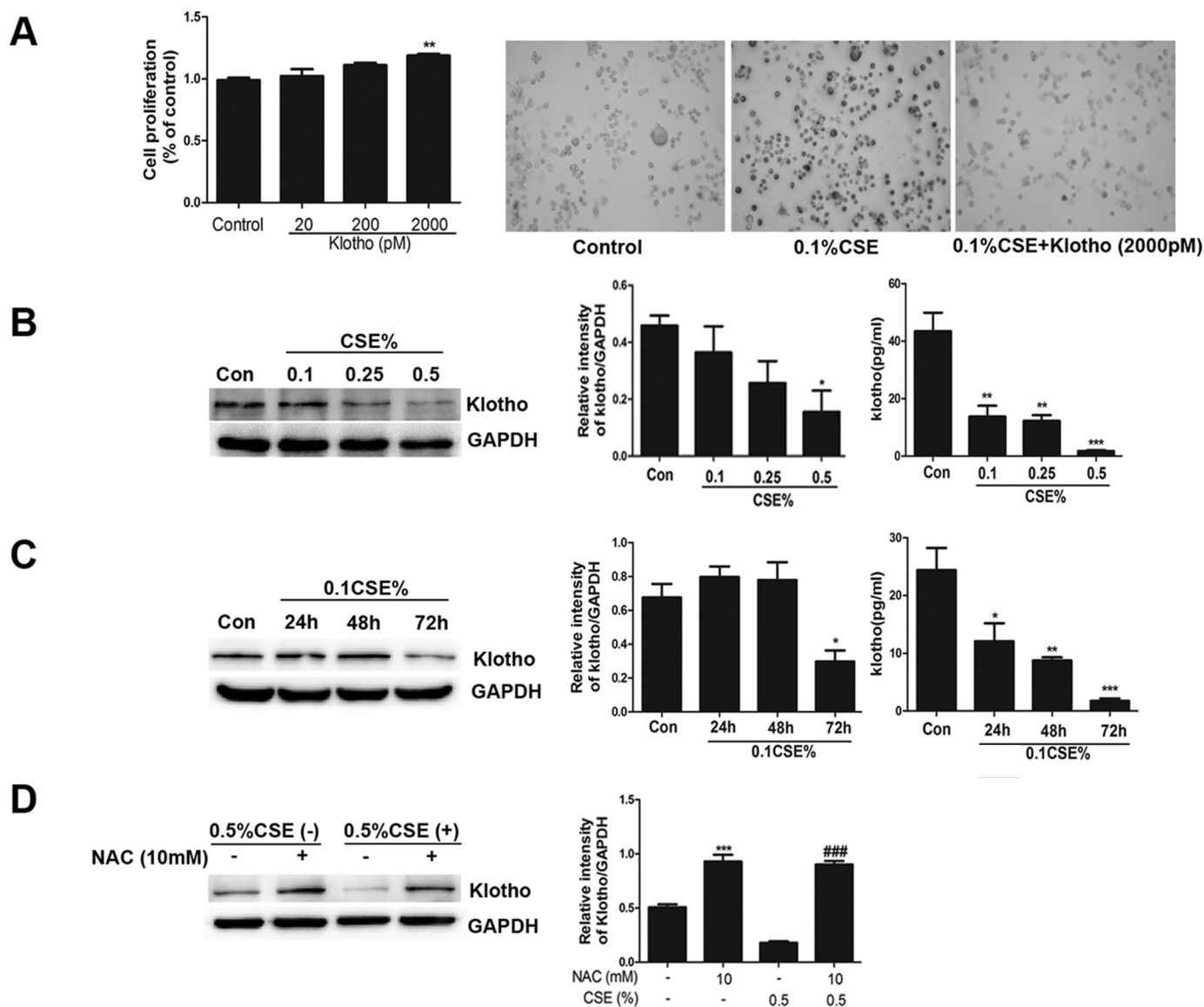


FIGURE 3. CSE decreased the expression and release of Klotho in MH-S cells. *A*, cells were pretreated with 20, 200, and 2000 pM Klotho protein in the presence or absence of 0.1% CSE for 24 h, respectively; cell proliferation was detected by CCK-8 assay, and senescence-associated β -gal activity was assessed. *B*, CSE decreased the protein levels of Klotho in MH-S cells at concentrations of CSE (0.1–0.5%) for 24 h. Relative densities (% of control) are shown of Klotho in MH-S cells at concentrations of CSE (0.1–0.5%) for 24 h. CSE decreased the release levels of Klotho in MH-S cells at concentrations of CSE (0.1–1.0%) for 24 h. *C*, 0.1% CSE decreased the protein levels of Klotho in MH-S cells at times of CSE treatment (24–72 h). Relative densities (% of control) are shown of Klotho in MH-S cells at times of CSE (24–72 h). 0.1% CSE decreased the release levels of Klotho in MH-S cells at times of CSE (24–72 h). *D*, cells were pretreated with 10 mM NAC in the presence or absence of 0.5% CSE for 24 h, and the protein expression of Klotho was examined by Western blotting. Data are the median of three independent experiments performed in triplicate. *, $p < 0.05$; **, $p < 0.01$, and ***, $p < 0.001$ compared with control values. ###, $p < 0.001$ compared with CSE.

measured using Image Lab2.0 software (Bio-Rad). GAPDH was used as a loading control.

RNA Isolation and Quantitative RT-PCR—Cultured cells were homogenized in TRIzol reagent (Invitrogen). Total RNA was isolated following the user's manual. Then 1 μ g of RNA was reverse-transcribed to cDNA using the PrimeScript RT-PCR kit (Takara, Kyoto, Japan). Quantitative real time RT-PCR was performed using SYBR Premix ExTaq II (Takara). Quantitative PCRs were performed using the SYBR Premix ExTaq kit (Takara). The primers used are presented in [supplemental Table 1](#). PCR conditions consisted of pre-denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 10 min.

ELISA—The culture medium was collected after treatment and centrifuged at 2500 rpm for 5 min to pellet the cells. The supernatant was then removed and stored at -20 °C for analysis. IL-6 and TNF- α levels in the supernatants were determined by ELISA from the respective duo-antibody kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

TransAM NF- κ B Assay—Nuclear extracts were prepared using the NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific, MA). The Active Motif TransAM NF- κ B ELISA kit (Active Motif) was used to determine the levels of p65 in nuclear extracts. In brief, 10 μ g of nuclear extract, diluted to 20 μ l, was added to the wells coated with oligonucleotides containing the NF- κ B consensus binding

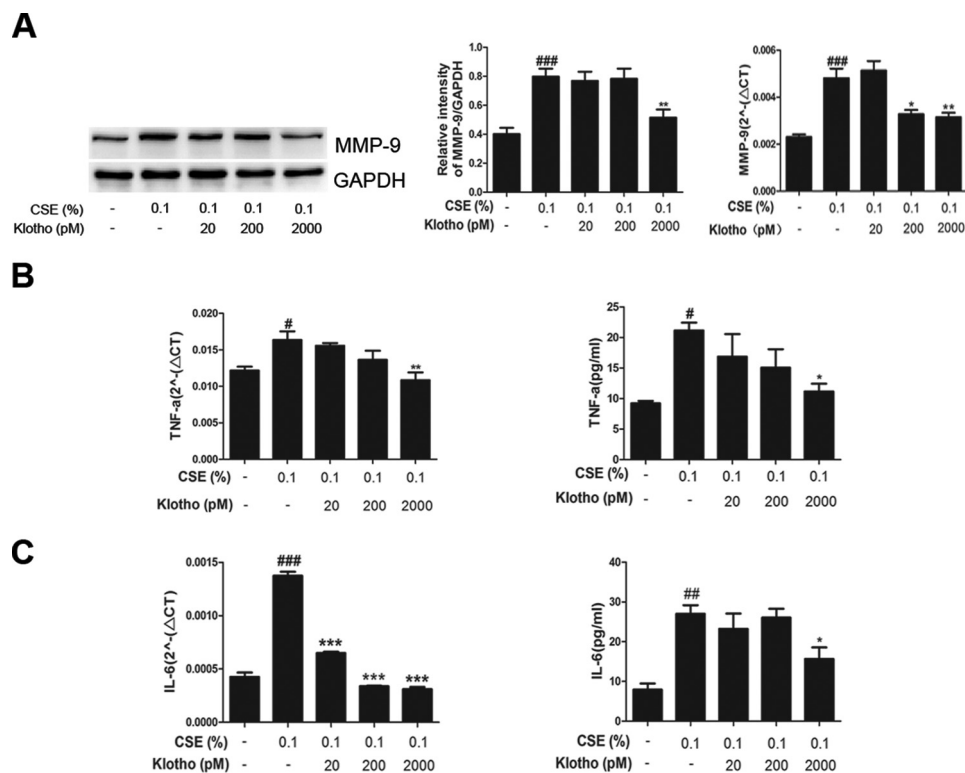


FIGURE 4. Klotho inhibited CSE-induced inflammation in MH-S cells. A, cells were pretreated with 20, 200, and 2000 pM Klotho protein for 30 min, respectively, and then incubated with 0.1% CSE for 24 h. The protein expression of MMP-9 was examined by Western blotting. The mRNA expression of MMP-9 was examined by real time PCR. B, cells were pretreated with 20, 200, and 2000 pM Klotho protein for 30 min, respectively, and then incubated with 0.1% CSE for 24 h. The mRNA expression of TNF- α was examined by real time PCR. The release levels of TNF- α were determined by ELISA. C, cells were pretreated with 20, 200, and 2000 pM Klotho protein for 30 min, respectively, and then incubated with 0.1% CSE for 24 h. The mRNA expression of IL-6 was examined by real time PCR. The release levels of IL-6 were determined by ELISA. Data are the median of three independent experiments performed in triplicate. *, $p < 0.05$; **, $p < 0.01$, and ***, $p < 0.001$ compared with CSE; #, $p < 0.05$; ##, $p < 0.01$; and ###, $p < 0.001$ compared with control.

site. The primary antibodies used to detect NF- κ B recognize an epitope on p65 that is accessible only when NF- κ B is activated and bound to its target DNA. After the addition of secondary antibodies conjugated with HRP and substrate, absorbance was read at 450 nm.

Immunoprecipitation—MH-S cells were treated with CSE (0.1, 0.25, and 0.5%) for 1 h at 37 °C; protein fraction was isolated, and Klotho was immunoprecipitated; Klotho antibody (1:80 dilution; Abcam) was added to 1 mg of protein to a final volume of 400 μ l and incubated for 1 h. 8 μ l of protein A/G-agarose beads (Invitrogen) were added to each sample and left overnight at 4 °C on a rocker. The samples were then centrifuged at 13,000 rpm at 4 °C for 5 min. The supernatant was discarded, and the beads were washed three times and then resuspended in 500 μ l of lysis buffer. For Western blots, 1 mg of the immunoprecipitated Klotho-agarose bead suspension were added to 50 μ l of 2 \times sample buffer, boiled, and resolved by SDS-PAGE as described above. Negative alone (beads only) was used as negative control. To demonstrate the interaction of Klotho protein with I κ B α , 50 μ g of immunoprecipitated Klotho were blotted against I κ B α .

Statistical Analysis—Statistical analysis was performed using SPSS 17.0 software. Results among groups were analyzed by one-way analysis of variance. Differences with a probability (p) value of less than 0.05 were considered statistically significant.

Results

Decreased Levels of Klotho in Alveolar Macrophages in Peripheral Lung Tissues of Smokers and Patients with COPD—To determine the levels of Klotho in alveolar macrophages, lung tissue sections from nonsmokers, smokers, and patients with COPD were analyzed for the expression of Klotho and CD68, a specific marker of macrophage. Immunohistochemical staining of fixed peripheral lung tissues demonstrated the decrease in the levels of Klotho in alveolar macrophages both in the lungs of smokers and in patients with COPD when compared with nonsmokers (Fig. 1).

Expression of klotho Is Correlated with an Increased Level of Inflammation and NF- κ B Activity in PBMCs of Smokers and Patients with COPD—As shown in Fig. 2A, klotho mRNA levels decreased in PBMCs from smokers and patients with COPD compared with nonsmokers. However, the levels of MMP-9, IL-6, and TNF- α significantly increased in the PBMCs of COPD patients as compared with those in healthy non-smokers (Fig. 2, B–D). NF- κ B activity was also significantly increased in PBMCs from smokers and patients with COPD compared with nonsmokers (Fig. 2E). klotho gene expression was significantly correlated with IL-6 and TNF- α gene expression (Fig. 2F). Furthermore, klotho gene expression correlated well with NF- κ B activity (Fig. 2F).

Effects of Klotho on Cell Proliferation and Cellular Senescence—MH-S cells were treated with Klotho at concentrations of 20, 200, and 2000 pM, respectively, for 24 h, and cell

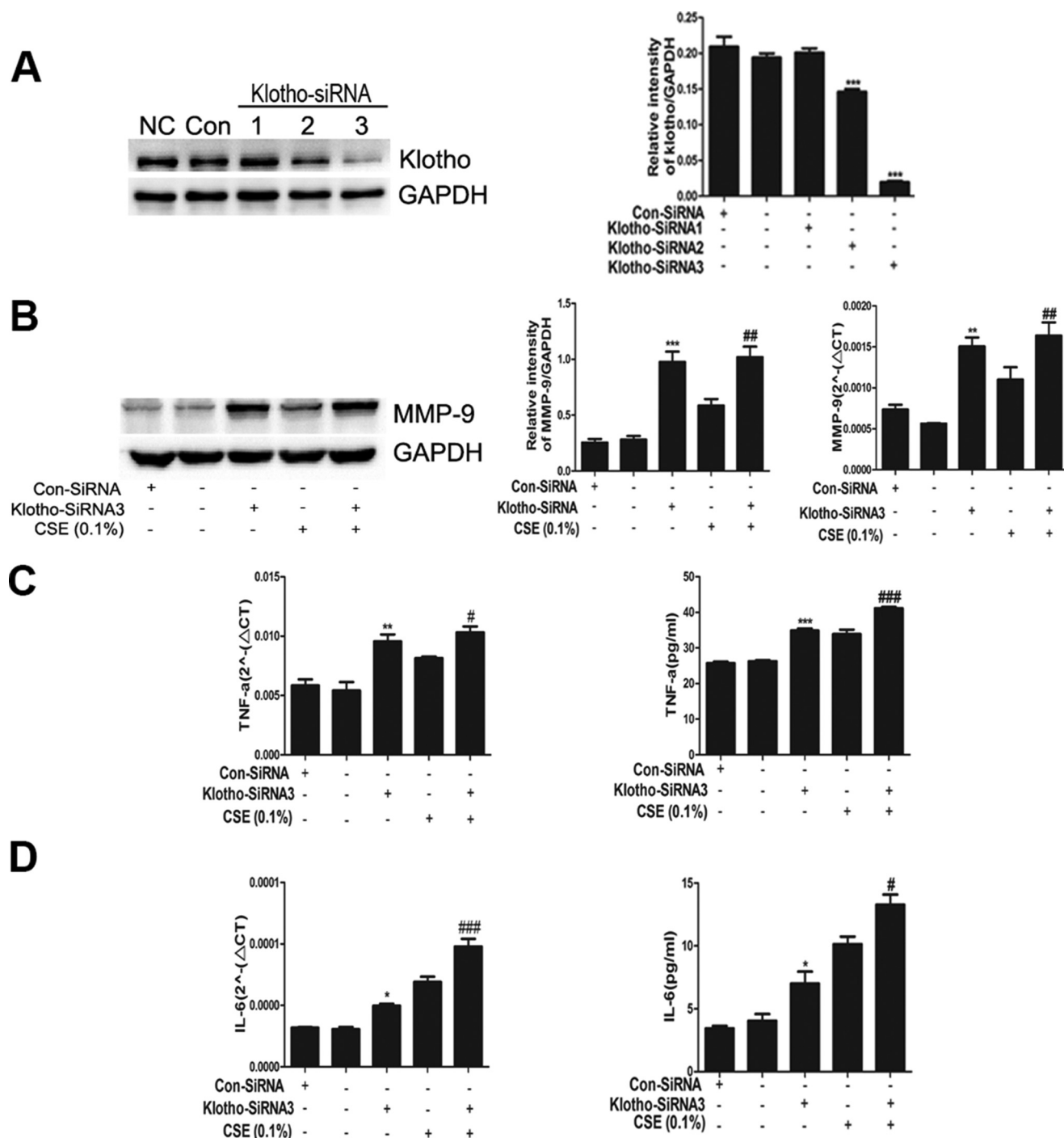


FIGURE 5. Knockdown of Klotho increased CSE-induced inflammation in MH-S. A, MH-S cells were transfected with negative control siRNA (NC siRNA) or *klotho* siRNA for 72 h. The protein expressions of Klotho were detected by Western blotting. B–D, *klotho* knockdown cells were stimulated with or without 0.1% CSE for 24 h, and the protein and mRNA expressions of MMP-9 and the mRNA expressions and release levels of TNF- α and IL-6 were examined by Western blotting, real time PCR, and ELISA, respectively. Data are the median of three independent experiments performed in triplicate. *, $p < 0.05$; **, $p < 0.01$, and ***, $p < 0.001$ compared with NC siRNA group; #, $p < 0.05$; ##, $p < 0.01$, and ###, $p < 0.001$ compared with CSE.

proliferation was determined by the CCK-8 kit. As shown in Fig. 3A, the results showed that Klotho at concentration of 2000 pM induced the growth of MH-S cells.

MH-S cells plated in 6-well chamber slides were treated with 0.1% CSE in the presence or absence of Klotho for 24 h. Cell senescence was detected the next day. The senescence-associated β -gal staining results showed that 0.1% CSE induced senescence of MH-S cells, whereas pretreatment

with 2000 pM Klotho rescued the senescence of MH-S cells (Fig. 3A).

Cigarette Smoke Decreases the Expression and Release of Klotho Protein Mediated by Reactive Oxygen Species in MH-S Cells—CSE (0.1, 0.25, and 0.5%) significantly decreased Klotho protein levels by MH-S cells in a concentration-dependent manner after 24 h of treatment (Fig. 3B). In addition, Klotho protein levels significantly decreased after 72 h of CSE (0.1%)

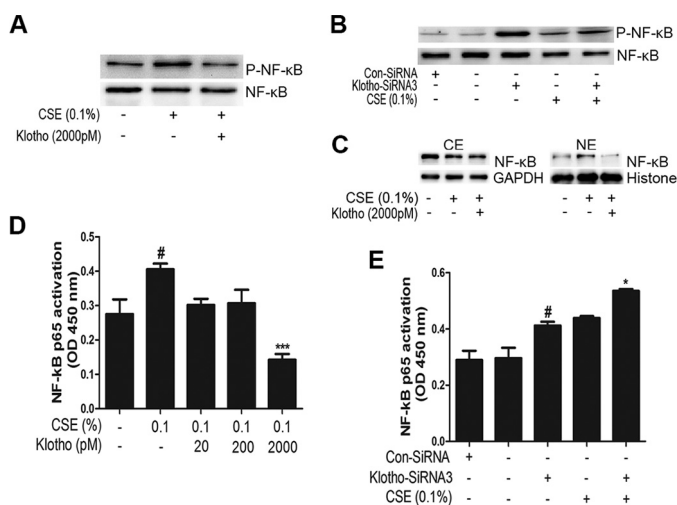


FIGURE 6. Klotho inhibited NF-κB phosphorylation, translocation to the nucleus, and DNA binding activity in CSE-induced MH-S cells. *A*, cells were pretreated with Klotho (2000 pM) for 1 h prior to stimulation with 0.1% CSE for 30 min. Western blot analysis was used to detect phosphorylation-NF-κB expression. *B*, *klotho* knockdown cells were stimulated with or without 0.1% CSE for 30 min; Western blot analysis was used to detect phosphorylation-NF-κB expression. *C*, cells were pretreated with Klotho (2000 pM) for 1 h prior to stimulation with 0.1% CSE for 5 min. Western blot analysis was used to detect NF-κB translocation to the nucleus. *CE*, cytoplasm extract; *NE*, nuclear extract. *D*, cells were pretreated with Klotho (2000 pM) for 1 h prior to stimulation with 0.1% CSE for 5 min. ELISA was used to detect nuclear NF-κB DNA binding activity. #, $p < 0.05$ compared with control values. ***, $p < 0.001$ compared with negative control siRNA group. *E*, *klotho* knockdown cells were stimulated with or without 0.1% CSE for 5 min. ELISA was used to detect nuclear NF-κB DNA binding activity. #, $p < 0.05$ compared with NC siRNA group; *, $p < 0.05$ compared with CSE. Data are the median of three independent experiments performed in triplicate.

treatment (Fig. 3C). We also examined the level of Klotho release in CSE-treated MH-S cells using ELISA. CSE treatment resulted in a marked decrease in Klotho release levels in MH-S cells after 24 h of CSE (0.1, 0.25, and 0.5%) treatment (Fig. 3B). A similar reduction in Klotho release levels was also observed in response to CSE (0.1%) treatment after 24, 48, and 72 h (Fig. 3C). We further searched the mechanism by which CSE modulates Klotho expression. *N*-Acetylcysteine (NAC) alleviated CSE-induced down-regulation of the Klotho protein (Fig. 3D). Taken together, CSE-induced generation of ROS suppressed Klotho expression in MH-S cells.

Klotho Suppresses CSE-induced Inflammation in MH-S Cells—To explore the effect of Klotho on the expression and release of proinflammatory cytokines, MH-S cells were pretreated with Klotho (20, 200, or 2000 pmol/liter) for 30 min, and then stimulated with CSE (0.1%) for 24 h. CSE strongly increased the protein and mRNA expression of MMP-9, as well as the mRNA expression and release of TNF- α and IL-6, whereas pretreatment of with Klotho significantly inhibited CSE-induced inflammatory mediator expression (Fig. 4).

Knockdown of Klotho Increases CSE-induced Inflammation in MH-S Cells—To further confirm the role of Klotho in CSE-induced inflammation in MH-S cells, cells were transiently transfected with negative control siRNA (NC siRNA) or Klotho siRNA (siRNA1, siRNA2, and siRNA3) for 72 h, and then the protein expression of Klotho was determined using Western blotting. As expected, the cells transfected with *klotho* siRNA showed a lower expression of the Klotho protein. Knockdown

efficiency of *klotho* siRNA3 was 90% as determined by Western blotting (Fig. 5A). Based on this result, we chose to use *klotho* siRNA3 in transfecting cells in the subsequent experiments. After knocking down endogenous *klotho*, cells were treated with CSE (0.1%) for 24 h. Knockdown of *klotho* or CSE treatment induced the inflammation of MH-S cells as compared with the control and negative control siRNA-transfected groups. There was a further increase in the level of inflammation in CSE-treated *klotho* siRNA-transfected cells as compared with CSE-treated cells alone (Fig. 5, B–D).

Klotho Inhibited the NF-κB p65 Pathway during CSE-induced Macrophage Inflammation in MH-S Cells—We examined the effect of Klotho on phosphorylation of NF-κB p65 in CSE-stimulated MH-S cells. MH-S cells were stimulated with CSE in the presence or absence of Klotho (at 2000 pmol/liter) for 30 min. Western blotting analysis showed that phosphorylation of NF-κB p65 markedly increased with CSE stimulation, whereas Klotho treatment significantly inhibited NF-κB p65 phosphorylation in CSE-stimulated MH-S cells (Fig. 6A).

Furthermore, we also established *klotho* knockdown MH-S cells using *klotho* siRNA3. NF-κB p65 phosphorylation induced by CSE in normal and Klotho-deficient cells was analyzed by Western blot. Our results showed that CSE-induced NF-κB p65 phosphorylation was activated in cells with siRNA-targeting *klotho* (Fig. 6B). Then we tested the effect of Klotho on NF-κB p65 nuclear translocation in CSE-stimulated MH-S cells. Alveolar macrophages were stimulated with CSE in the presence or absence of Klotho (at 2000 pmol/liter) for 5 min. Western blotting analysis showed that NF-κB p65 nuclear translocation was promoted by CSE stimulation, whereas Klotho treatment significantly inhibited NF-κB p65 nuclear translocation in CSE-stimulated MH-S cells (Fig. 6C). We then examined the effect of Klotho on NF-κB p65 DNA binding activity in CSE-stimulated MH-S cells. Alveolar macrophages were stimulated with CSE in the presence or absence of Klotho (at 20, 200, or 2000 pmol/liter) for 5 min. Analysis by TransAM NF-κB assay showed that NF-κB p65 DNA binding activity significantly increased with CSE treatment, whereas Klotho treatment significantly suppressed NF-κB p65 DNA binding activity in CSE-stimulated MH-S cells. We also established *klotho* knockdown MH-S cells using *klotho* siRNA3. Our results showed that CSE-induced NF-κB p65 DNA binding activity was activated in cells with siRNA-targeting *klotho* (Fig. 6, D and E).

To further confirm whether Klotho regulates inflammation expression through the NF-κB pathway, we employed the NF-κB inhibitor JSH23 to block the NF-κB activity in *klotho*-transfected MH-S cells. As shown in Fig. 7, A–C, the NF-κB inhibitor reversed the up-regulated inflammatory expression after *klotho* knockdown.

Klotho Interacts with the NF-κB Inhibitor IκBα—We further examined whether Klotho protein is physically associated with the inhibitor of NF-κB p65, IκBα. With the use of immunoprecipitation of Klotho and Western blotting of IκBα, IκBα degradation significantly increased in whole extracts of MH-S cells with CSE treatment (Fig. 7D).

Effect of Dexamethasone on the Expression and Release of Klotho in MH-S Cells—Dexamethasone increased the expression of Klotho protein in MH-S cells following CSE stimulation

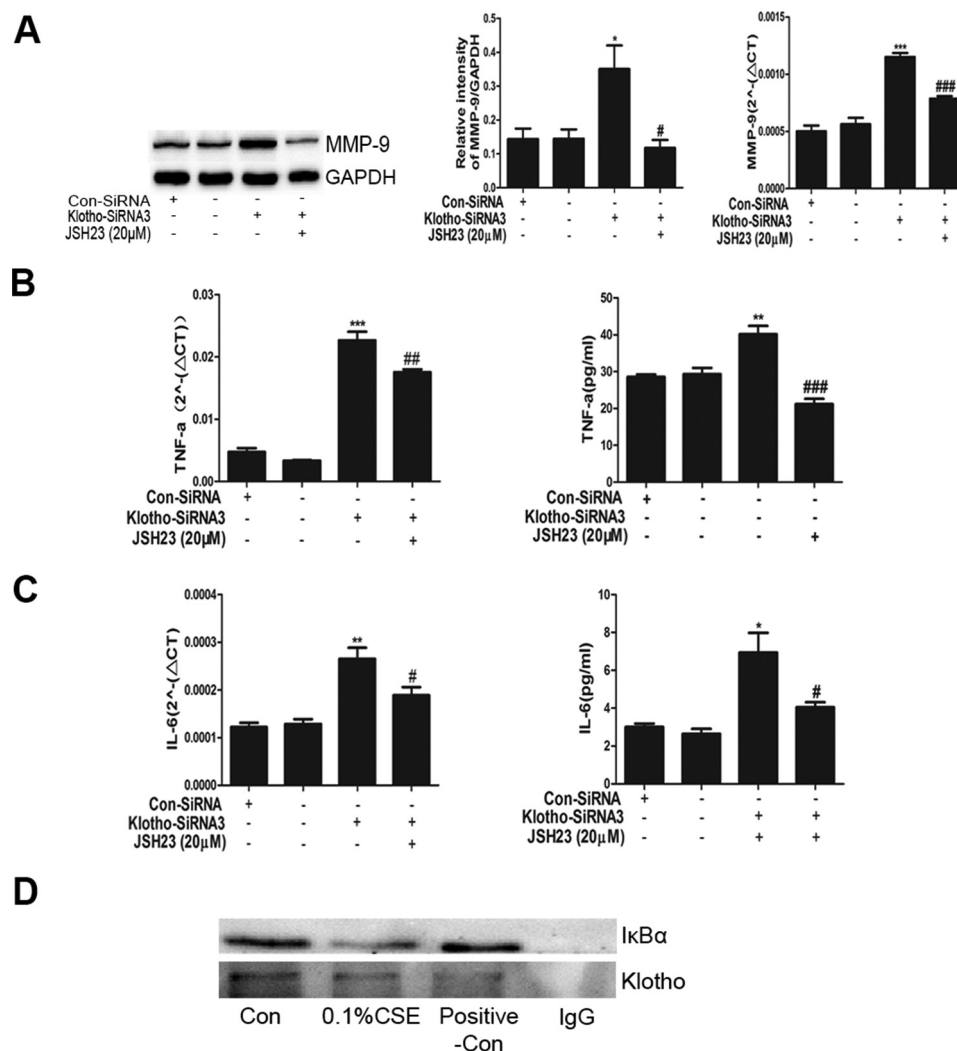


FIGURE 7. NF-κB inhibitor (JSH23) reversed the up-regulation of inflammation expression in Klotho knockdown cells and Klotho interacts with the NF-κB inhibitor IκBα in MH-S cells. *A*, Klotho knockdown cells were treated with JSH23 for 24 h. The protein expressions of MMP-9 were detected by Western blotting. The mRNA expression of MMP-9 was detected by PCR. *B*, Klotho knockdown cells were treated with JSH23 for 24 h. The mRNA expression of TNF-α was detected by real time PCR. The release levels of TNF-α were determined by ELISA. *C*, Klotho knockdown cells were treated with JSH23 for 24 h. The mRNA expression of IL-6 was detected by real time PCR. The release levels of IL-6 were detected by ELISA. *D*, Klotho was immunoprecipitated from MH-S cells treated with CSE (0.1%) for 1 h. Levels of IκBα were analyzed by immunoblotting. Then IκBα was immunoprecipitated from MH-S cells treated with CSE (0.1%) for 1 h. Levels of Klotho were analyzed by immunoblotting. CSE (0.1%) significantly decreased IκBα levels in the Klotho-IκBα complex. An equal amount (1 mg) of immunoprecipitated protein was used for Western blots. Data are the median of three independent experiments performed in triplicate. *, $p < 0.05$; **, $p < 0.01$, and ***, $p < 0.001$ compared with negative control siRNA group. #, $p < 0.05$; ##, $p < 0.01$, and ###, $p < 0.001$ compared with Klotho siRNA group. Con, control.

(Fig. 8A). Dexamethasone also significantly increased Klotho protein expression in MH-S cells, as measured by ELISA (Fig. 8B).

Discussion

In this study, we have shown that there is a reduced level of Klotho in alveolar macrophages in the lungs and PBMCs of COPD patients. *In vitro*, CSE caused the inhibition of both expression and release of Klotho in a time- and concentration-dependent manner in MH-S cells, which is through the NF-κB pathway and coincided with increased expression of MMP-9, TNF-α, and IL-6. These results indicate that cigarette smoking might promote the airway inflammation through the inhibition of Klotho by alveolar macrophages in COPD formation.

In our study, at the concentration of 2000 pM, Klotho induced the growth of MH-S cells and suppressed MH-S cells senes-

cence, which indicates that it may exert an effect on cell proliferation through the inhibition of senescence. We demonstrated that the protein expression and release level of Klotho were significantly decreased in MH-S cells treated with CSE in a time- and concentration-dependent manner. This notion is consistent with a previous study; Klotho is down-regulated by inflammatory cytokines such as TWEAK or TNF-α that decreased Klotho gene transcription in renal tubular cells (22, 23). Klotho is an important protein involved in the regulation of proinflammatory cytokine release, stress resistance, metabolism, senescence, and aging (24–27), all of which are linked to the pathogenesis of COPD (5, 6, 13, 29, 38). Therefore, we hypothesized that CSE-mediated reduction in Klotho may in part be responsible for the increased inflammatory response in smokers and patients with COPD. It is well known that CSE-induced oxidative stress is responsible for proinflammatory

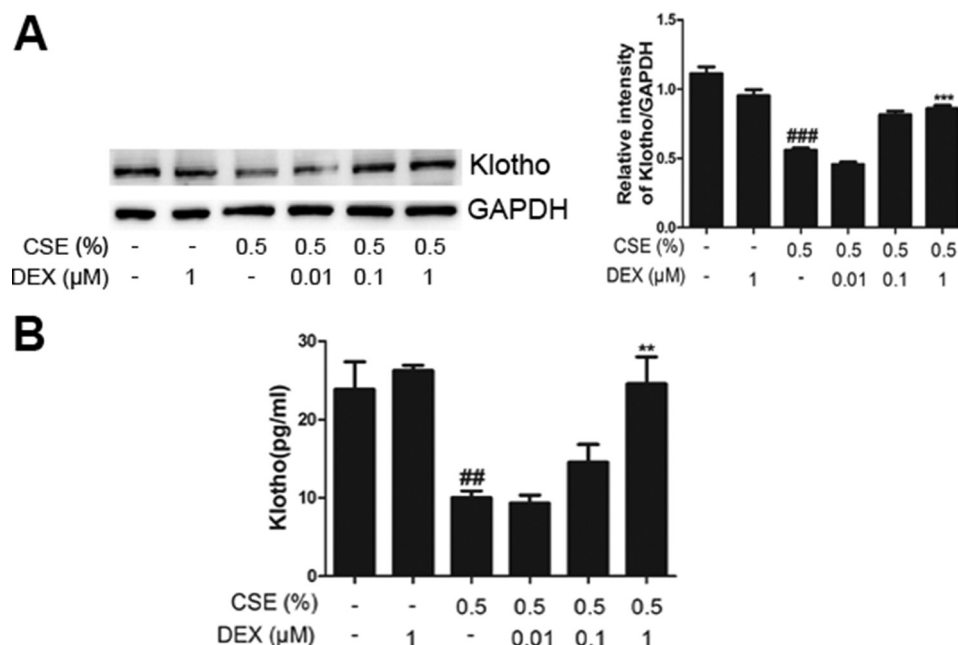


FIGURE 8. Effect of dexamethasone on the protein expression and release level of Klotho in MH-S cells. *A*, cells were pretreated with dexamethasone (*Dex*) (0.01, 0.1, and 1 μ M) for 1 h prior to stimulation with 0.5% CSE for 24 h. The protein expressions of Klotho were detected by Western blotting. *B*, cells were pretreated with dexamethasone (0.01, 0.1, and 1 μ M) for 1 h prior to stimulation with 0.5% CSE for 24 h. The release levels of Klotho were detected by ELISA. Data are the median of three independent experiments performed in triplicate. ##, $p < 0.01$, and ###, $p < 0.001$ compared with control. **, $p < 0.05$, and ***, $p < 0.001$ compared with CSE.

cytokine release in the lung (30). Our results showed that NAC was effective in alleviating CSE-induced down-regulation of Klotho protein. The possible mechanism is that NAC may enhance the Klotho expression through the suppression of CSE-induced ROS.

Alveolar macrophages are considered as important cells in perpetuating the inflammatory response of CSE in COPD (5, 13, 31). Klotho is an anti-inflammatory modulator in the kidney. In a mouse model of diabetes, Klotho depletion contributes to increased inflammation (32). A previous study showed that the lack of Klotho protein may indirectly increase the expression of MMP-9 in the lung of *klotho* mice (15). Therefore, we examined the specific effect of Klotho on CSE-induced inflammation in MH-S cells. We studied the effect of exogenous Klotho on CSE-induced inflammation by treatment with Klotho protein and knocking down Klotho in MH-S cells. The results showed that Klotho protein suppressed the expression and release levels of MMP-9, TNF- α , and IL-6, whereas knockdown of endogenous Klotho augmented the CSE-stimulated inflammation (MMP-9, TNF- α , and IL-6). The present findings emphasize the importance of Klotho in the regulation of inflammation by alveolar macrophages. These facts are consistent with the anti-inflammatory effects of Klotho that were observed in the previous study (32).

In this study, we demonstrated that Klotho inhibits the inflammatory mediators' (MMP-9, TNF- α , and IL-6) expression through the NF- κ B pathway. However, previous studies have shown that Klotho can regulate the pathways of insulin/IGF-1 and Wnt and the activity of multiple ion channels (33); it is also possible that these pathways may also be involved in the regulation of the cytokines above (MMP-9, TNF- α , and IL-6). The transcription factor NF- κ B RelA was required for suppres-

sion of Klotho gene transcription (23). NF- κ B is also an important transcriptional factor in the regulation of inflammatory genes in the airways of people with COPD (34). Based on these findings, we hypothesized that NF- κ B is likely to be involved in the underlying mechanism by which Klotho inhibits CSE-stimulated macrophage inflammation. NF- κ B is a key transcription factor, widely distributed in most cell types. It mainly consists of dimers of the two subunits p50 and p65 (RelA), and the p65 protein is the key transcriptionally active component of NF- κ B. In non-stimulated cells, it exists in the cytoplasm in an inactive form associated with an inhibitory protein called I κ B. The release of the inhibitory I κ B subunit from the complex, followed by phosphorylation and translocation of the dimer to the nucleus, leads to the activation of NF- κ B (35). In the nucleus, it regulates the transcription of various genes (35, 36). In this context, we showed that CSE significantly activated NF- κ B p65 phosphorylation, nuclear translocation, and DNA binding activity. Exogenous Klotho obviously attenuated the CSE-induced phosphorylation, nuclear translocation, and DNA binding activity of NF- κ B p65, whereas knockdown of Klotho strikingly increased the effect of CSE on the NF- κ B pathway in MH-S cells. We further affirmed the above results by showing that the NF- κ B inhibitor, JSH23, ameliorated the up-regulated inflammation expression after knocking down Klotho. Our results indicate that Klotho inhibits NF- κ B-mediated inflammatory mediator release and possibly attenuates inflammation in response to inflammatory stimulus.

I κ B α is a major regulator of NF- κ B. This study also showed that the interaction of I κ B α with Klotho and the disruption of this complex and NF- κ B activation by exposure to CSE may explain the sustained proinflammatory cytokine release, sug-

gesting that reduced levels of Klotho by CSE would have an impact on degradation of I κ B α and activation of NF- κ B p65.

Our findings regarding cigarette smoke-mediated reduction in Klotho (anti-aging protein) and augmented lung inflammatory response have special implications in lung senescence, as the role of senescence has been recently implicated in emphysema (28, 37). Therefore, it is tempting to speculate that cigarette smoke-induced inflammation and senescence of lung cells via Klotho and the NF- κ B axis.

In this study, dexamethasone treatment increased the expression and release levels of Klotho in MH-S cells. Our data also showed that dexamethasone did not affect the transcriptional expression of Klotho, which means dexamethasone may have a post-transcriptional regulation on Klotho expression (data were not shown). However, some of the patients with COPD were receiving inhaled steroids. Therefore, it is likely that, once initiated, the alterations of Klotho may not be fully reversible (irreversible epigenetic events), which in turn might be one contributor to the persistence of various inflammatory changes observed even after cessation of smoking.

A potential weakness of this study is that the precise mechanism whereby CSE alters the levels of Klotho is not known. Further studies are required to understand the mechanism of CSE-mediated down-regulation of Klotho and its involvement in chronic inflammatory and injurious processes in the lung using genetic gain and loss of function, and whether up-regulation or genetic modifications of Klotho can attenuate such processes in animal models of COPD.

In conclusion, we have shown for the first time that the level of Klotho protein was decreased in alveolar macrophages in peripheral lung tissue of smokers and patients with COPD and in PBMC. Our data show for the first time that cigarette smoke decreased Klotho levels by CSE-mediated mechanisms in alveolar macrophages. Klotho treatment inhibited CSE-mediated inflammation, whereas knockdown of Klotho augmented CSE-mediated inflammation, suggesting that Klotho regulates CSE-mediated induction of MMP-9, TNF- α , and IL-6. Overall, our study provides novel data on an important molecular mechanism by which Klotho regulates cigarette smoke-driven NF- κ B-dependent inflammation in alveolar macrophages.

Author Contributions—X. Y. supervised the project. L. L. and Y. W. designed the study. L. L. and Y. W., and W. G. conducted the experiments. L. L., C. Y., S. Z., M. H., and H. Z. analyzed data; and L. L. and X. Y. wrote the paper. All authors read the paper and contributed to its final form.

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